**Herbal formula BWBDS alleviates polymicrobial sepsis-induced liver injury via increasing the gut microbiota *Lactobacillus johnsonii* and regulating macrophage anti-inflammatory activity in mice 1 [[1]](#footnote-1)**

**Abstract** Sepsis-induced liver injury (SILI) is an important cause of septicemia deaths. BaWeiBaiDuSan (BWBDS) was extracted from a formula of *Panax ginseng C. A. Meyer, Lilium brownie F. E. Brown ex Miellez var. viridulum Baker, Polygonatum sibiricum Delar. ex Redoute, Lonicera japonica Thunb., Hippophae rhamnoides Linn, Amygdalus Communis Vas, Platycodon grandiflorus (Jacq.) A. DC., and Cortex Phelloderdri*. Herein, we investigated whether the BWBDS treatment could reverse SILI by the mechanism of modulating gut microbiota. BWBDS protected mice against SILI, which was associated with promoting macrophage anti-inflammatory activity and enhancing intestinal integrity. BWBDS selectively promoted the growth of *Lactobacillus johnsonii* (*L. johnsonii*) in cecal ligation and puncture (CLP) treated mice. FMT treatment indicated that gut bacteria correlated with sepsis and was required for BWBDS anti-sepsis effects. Notably, *L. johnsonii* significantly reduced SILI by promoting macrophage anti-inflammatory activity, increasing IL-10+ M2 macrophage production and enhancing intestinal integrity. Furthermore, heat inactivation *L. johnsonii* (HI-*L. johnsonii*)treatment promoted macrophage anti-inflammatory activity and alleviated SILI. Our findings revealed BWBDS and gut microbiota *L. johnsonii* as novel prebiotic and probiotic that may be used to treat SILI. The potential underlying mechanism was at least in part, via *L. johnsonii*-dependent immune regulation and IL-10+M2 macrophage production.

**Keywords:** BWBDS; Sepsis-induced liver injury; Network pharmacology; 16S PacBio SMRT sequencing; macrophages; IL-10.

1. **Introduction**

Sepsis is widely perceived as a life-threatening organ dysfunction that is caused by a dysregulated host response to infection[1](#_ENREF_1). Antibiotics is an essential component of initial therapy in sepsis[2](#_ENREF_2). However, inappropriate use of antibiotics can lead to multidrug-resistant infections and death[3](#_ENREF_3). Therefore, it is necessary to discover novel targeted therapeutic strategies. The gut-liver axis refers to the bidirectional relationship between the microbiota, metabolic and immune crosstalk between the gut and liver, connected in a bidirectional fashion by the portal vein and biliary tree[4](#_ENREF_4). In the last 20 years, interactions and organ-organ communication between key organs (such as the liver and gut) during sepsis have been well understood[5](#_ENREF_5). However, sepsis-induced liver injury (SILI) is related to adverse clinical outcomes[6](#_ENREF_6). Therefore, the pathogenesis of the gut and the liver injury are currently being investigated during sepsis, treatments that create physical barriers between the gut and the liver offer more effective prevention and therapeutic strategies.

Previous studies have found that gut microbiota dysbiosis is related to disruption of intestinal barrier function, immune function and liver damage[7-9](#_ENREF_7). Approaches that reshape the intestinal flora have therefore been used for the prevention and treatment of liver injury[10](#_ENREF_10),[11](#_ENREF_11). Microbial metabolites affect this progression[12](#_ENREF_12). Diet is a critical factor that influences host metabolism by regulating the intestinal microbiota[13](#_ENREF_13). Study has indicated that using prebiotics and probiotics to modulate the gut microbiota may improve gut integrity, host metabolism and liver injury[14](#_ENREF_14). Nevertheless, the complex interactions between diet, prebiotics and the gut microbiota need further studies.

BaWeiBaiDuSan (BWBDS) is a hospital prescription. Clinically, BWBDS has been used to treat postoperative infection successfully. BWBDS including such as *Panax ginseng C. A. Meyer*, *Lilium brownie F. E. Brown ex Miellez var. viridulum Baker*,and *Amygdalus Communis Vas* commonly are usedas Western dietary supplement[15-17](#_ENREF_15),and the others (*Polygonatum sibiricum Delar. ex Redoute, Lonicera japonica Thunb., Hippophae rhamnoides Linn, Platycodon grandiflorus (Jacq.) A. DC., and Cortex Phelloderdri*) are traditional medicines in China and other Asian countries[18](#_ENREF_18). The main ingredients from BWBDS possess various physiological activities, including anti-bacterial and anti-inflammatory[19](#_ENREF_19). Network pharmacology showed that these mechanisms may influence the response to molecule of bacterial origin and regulation of inflammatory response. Ginseng polysaccharides have been demonstrated to improve intestinal metabolism and modulate the gut microbiota[20](#_ENREF_20). In addition, they are found to be responsible for the immunomodulatory functions and regulate liver damage[21-23](#_ENREF_21). Nevertheless, the therapeutic potential of BWBDS in polymicrobial sepsis is unclear. In this study, we used cecal ligation and puncture (CLP) model to investigate the potential of BWBDS for sepsis treatment and investigated the mechanisms related to the gut-liver axis.

1. **Materials and Methods**

*2.1. Animal experiments*

C57BL/6 mice, male, 6-8 weeks were used and housed three to four mice in independently vented at the animal facility of the State Key Laboratory of Quality Research in Chinese Medicine, Macau University of Science and Technology.

CLP model was induced according to the method described previously[11](#_ENREF_11). For BWBDS or cefoxitin sodium (CS) treatment, mice were administered BWBDS (1500mg/kg) by oral gavage twice daily for two weeks then undergoing CLP and injection of CS (500 mg/mice) for three days post CLP. For antibiotic (ABX) treatment, mice were administered vancomycin (100 mg/kg), neomycin sulfate (200 mg/kg), metronidazole (200 mg/kg), and ampicillin (200 mg/kg) by oral gavage once daily for 5 days. For *Lactobacillus johnsonii* (*L. johnsonii*) treatment, mice were treated with *L. johnsonii* (5×108 CFU/day) by oral gavage for 1week before establishing CLP. Control mice received the equivalent volume of MRS medium. For experiment involving clodronate liposomal (Clo-Lipo), mice were intraperitoneally injected with 200 μl Clo-Lipo (Yeasen, Shanghai, China) 24 h before establishing CLP model. Control mice received the equivalent volume of empty liposomes (Lipo) (Yeasen, Shanghai, China). For experiment involving anti-IL-10R mAb, mice were intragastrically administrated with anti-IL-10R mAb (cat#no:112713, Biolegend, California, USA‎) 0.5 mg/mice for 3 times at 3-day intervals on day 0 after antibiotic cocktail treatment. For experiment involving heat inactivation *L. johnsonii* (HI-*L. johnsonii*), mice were treated with HI-*L. johnsonii* (5×108 CFU/day) by oral gavage for 1week before establishing CLP.

*2.2. Fecal microbiota transfer (FMT)*

The modified method described previously was used to perform FMT[24](#_ENREF_24). Briefly, fecal samples were collected on day 14 after administration BWBDS. the gut microbiota was eliminated by oral gavage antibiotics cocktail in mice for 5 days. Then feces pellets prior to suspension and homogenization in PBS at 0.125 g/ml, and fecal supernatant after centrifugation was treated daily to mice via oral gavage for 3 days, then subjected to CLP.

*2.3. Flow cytometry and antibodies*

The spleen, peritoneal macrophages (PMs), and polarized peripheral blood mononuclear cells (pPBMC) were harvested and lysed with RBC lysing buffer (Biolegend, 420301). Cells were incubated with an Fc receptor blocker (CD16/32, eBioscience) to reduce nonspecific antibody binding prior to adding staining antibodies. For live/dead cell separation 7-AAD staining was used. Cells were labeled with APC anti-mouse F4/80, PerCP-Cyanine5.5 anti-mouse CD45, FITC anti-mouse CD11b, Brilliant Violet 421 anti-mouse CD206, Brilliant Violet 650 anti-mouse CD80 (Biolegend, clone BM8, clone 30-F11, clone M1/70, clone C068C2, and clone 16-10A1 respectively, USA). For intracellular labeling, cells were stimulated with LPS (10 μg/ml), 3 μg/ml brefeldin A for 5 hour at 37°C[25](#_ENREF_25). The cells were stained with Brilliant Violet 605 anti-mouse IL-10, PE/Dazzle 594 tumor necrosis factor (TNF)-α (Biolegend, clone JES5-16E3 and clone 506346, respectively, USA), and PE anti-mouse inducible nitric oxide synthase (iNOS) (eBioscience, clone CXNFT, respectively, USA) and then analyzed using an CytoFLEX flow cytometer (BECKMAN COULTER, USA). The data were analyzed by using FlowJo software (V.10.4, FlowJo, USA)

*2.4. Fecal DNA extraction and 16S rRNA sequencing*

The preparation of fecal sample and the sequencing of 16S rRNA were conducted as previously described[20](#_ENREF_20). Polymerase chain reaction (PCR) was used to amplify fecal DNA samples via using barcoded primer pairs targeting 16S rRNA gene. PCR amplicons were sequenced using the PacBio Sequel System. The resulting bacterial sequence fragments were analyzed in QIIME 2.

*2.5. Statistical analysis*

Results were represented as the mean ± SEM, p<0.05 is considered statistically significant. Data were performed by unpaired, two-tailed Student’s t test, log-rank (Mantel Cox) test, Mann-Whitney test, one-way ANOVA, and two-way ANOVA (GraphPad Prism software).

1. **Results**

*3.1. Network pharmacology predicts the possible herb-compound-target-pathway interaction associating with the anti-sepsis effect of BWBDS*

We have performed qualitative and quantitative analysis of BWBDS by LC/MS/MS analysis and the results were shown in (Supporting Information Fig. S1A and B, Supporting Information Table S2). To explore the multiple components, multiple targets, and various pathways of BWBDS, network pharmacology was applied to predict possible mechanisms. 74 bioactive components were predicted to act on 486 sepsis-related targets (Supporting Information Table S3 and Supporting Information Fig. S2A). The top 10 genes of BWBDS are PIK3CA (phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha), AKT1 (AKT serine/threonine kinase 1), PIK3R1 (phosphoinositide-3-kinase regulatory subunit 1), APP (amyloid precursor protein), MAPK1 (mitogen-activated protein kinase 1), EP300 (E1A binding protein P300), MAPK3 (mitogen-activated protein kinase 3), STAT3 (signal transducer and activator of transcription 3), UBC (ubiquitin C) and SRC (sarcoma) (Supporting Information Fig. S2B). According to the results of KEGG (Kyoto Encyclopedia of Genes and Genomes) (Supporting Information Fig. S2C and D) and GO enrichment analysis (Supporting Information Fig. S3), several important validation related pathways, such as TNF and interleukin-17 (IL-17), were predicted to be the mechanism of BWBDS. The results also showed that BWBDS were related to PI3K-Akt (phosphatidyl-inositol 3-kinase/serine-threonine kinase), MAPK (Mitogen-Activated Protein Kinase 3), SRC and other upstream inflammatory signal transduction pathways. These mechanisms may influence the response to molecule of bacterial origin and regulation of inflammatory response.

*3.2. BWBDS treatment alone protects mice against SILI*

To determine if BWBDS could protect mice against CLP-induced sepsis, we treated CLP mice with BWBDS before the surgery for two weeks and positive medicine CS for three days when the surgery was finished (Supporting Information Fig. S12A). As shown in (Supporting Information Fig. S4A and B), BWBDS or CS-treated animals exhibited no significant weight loss before CLP, but significantly slowed weight loss compared with CLP control mice at 24h. In addition, the treatment group animals exhibited significantly longer survival and lower clinical scores compared with CLP control mice ((Fig. 1A and Supporting Information Fig. S4C).

Excessive cytokine production by immune cells is a major cause of SILI[26](#_ENREF_26), we therefore tested whether BWBDS could reduce cytokines secretion during septic inflammation. We found the secretion of proinflammatory cytokines interleukin-6 (IL-6), interleukin-1β (IL-1β), and tumor necrosis factor-alpha (TNF-α) were markedly decreased, and anti-inflammatory cytokines IL-10 was significantly upregulated in mice serum upon BWBDS treatment (Fig. 1B-E). In agreement with these findings, the mRNA levels of liver inflammation markers (IL-6, TNF-α, and IL-1β) were markedly reduced in BWBDS-treated mice compared with CLP control mice. Other key inflammatory factors, such as C-C motif chemokine ligand (CCL2, CCL3, CCL7), C-X-C motif chemokine ligand (CXCL1, CXCL10) were also reduced after BWBDS treatment (Fig. 1F). Histological analysis indicated that there were fewer pathological changes in livers from BWBDS-treated mice compared with CLP control mice (Fig. 1G and H). BWBDS treatment also markedly improved the damage of kidney and lung, and tissue necrosis in the cecal after subjecting to CLP (Supporting Information Fig. S4D-J).

We found CS, BWBDS, and CS plus BWBDS treatment could significantly decrease F4/80+CD11b+CD45+ macrophages proportion in the liver, and BWBDS showed more effective than CS (Fig. 1I). To determine if BWBDS could reduce the degree of liver injury during sepsis, we next tested the serum transaminase levels [alanine aminotransferase (ALT) and aspartate aminotransferase (AST)]. We found that ALT and AST in BWBDS-treated mice were markedly diminished compared with those in CLP control mice at 24 h after CLP, while no difference was observed compared with sham operation mice, CS-treated mice and BWBDS plus CS-treated mice (Fig. 1J and K).

Previous studies have indicated that a sepsis decreases expression of intestinal tight junction proteins (e.g., zonula occludens-1 (ZO-1), occludin) as well as disrupts gut barrier integrity, resulting in migration of bacterial lipopolysaccharide (LPS) into the blood and causing inflammation[27](#_ENREF_27). Moreover, disruption of gut tight junctions may be an important pathway for sepsis-induced multiple organ dysfunction[28](#_ENREF_28). To determine if BWBDS could improve gut-barrier function. We next tested the expression of intestinal tight junction proteins. We observed that the BWBDS treatment markedly increased colonic mRNA levels of ZO-1 and occludin than CLP control mice, while no difference was observed compared with CS-treated mice and BWBDS plus CS-treated mice (Fig. 1L and M).

It is known that the side effects of antibiotics CS are associated with poor prognosis of clinical sepsis. Our result indicated that BWBDS alone already had a better effect than CS. Our purpose to set up the CS plus BWBDS treatment group was to verify if the conventional CS treatment could attenuate the beneficial effect of BWBDS. When BWBDS combined with CS treatment, there was no significant difference when compared with BWBDS alone group, CS did not decrease the anti-sepsis effect of BWBDS, so BWBDS could be used singly or combined with CS to treat sepsis. In conclusion, our results indicated that BWBDS treatment alone may reverse SILI.

*3.3. The anti-SILI effect of BWBDS treatment is related to promoting the anti-inflammatory activation of macrophages*

Previous studies have shown that conversion of M1 to M2 macrophages and vice versa during responses to infection, wound healing, and cancers[29](#_ENREF_29). To determine the effects of the BWBDS therapy on the macrophages, we analysed the changes of macrophages in the PMs, spleen and pPBMC using flow cytometry. We observed that total macrophages (F4/80+CD11b+CD45+) and M1 macrophages (CD80+F4/80+CD11b+) decreased, meanwhile,M2 macrophages (CD206+F4/80+CD11b+) increased in BWBDS treated mice when compared with CLP control mice (Fig. 2A-C). The production of functional cytokines, TNF-α and iNOS among M1 macrophages also decreased (Fig. 2D and E). In addition, we also observed the upregulation of IL-10 among M2 macrophages (Fig. 2F) (Supporting Information Fig. S5). Our results revealed that BWBDS treatment may have effect on SILI via activating M2 macrophages and suppressing the function of M1 macrophage.

*3.4. Distinct gut microbiota composition is present in BWBDS-treated mice and CLP control mice*

To investigate whether the composition of the gut microbiome is associated with anti-sepsis effect, the fecal samples were tested by enterobacterial repetitive intergenic consensus (ERIC)-PCR and 16S PacBio SMRT sequencing. The results of ERIC-PCR showed that the composition of intestinal flora was different in different groups (Supporting Information Fig. S6). The alpha diversity showed that the richness and diversity of gut microbiome in BWBDS treated mice were higher than the CLP control mice (Fig.3A and B). The relative abundance of *Firmicutes* and *Firmicutes/Bacteroidetes* ratio were significantlyincreased (Fig. 3C, 3D), while the relative abundance of *Bacteroidetes* as well as the *Proteobacteria* were decreased in BWBDS treated mice compared with CLP control mice (Fig. 3E and 3F). The beta diversity (abund\_jaccard) showed that the composition of intestinal microbiota in BWBDS treatment group were different compared with CLP control group, CS treatment group, and CS plus BWBDS treatment group (Fig. 3G). Moreover, we found the abundance of *Lactobacillus* in genus and species were increased in BWBDS-treated mice compared with CLP control mice (Fig. 3H and I). There were also differences in the abundance of *Lactobacillus* species between CS treatment group and BWBDS plus CS treatment group (Supporting Information Fig. S7). These results indicated that BWBDS-treated mice had a unique intestinal microbiome composition compared with the CLP control mice and CS-treated mice. BWBDS treatment may increase probiotics and decrease harmful bacteria, which may be helpful for the anti-SILI effect of BWBDS.

*3.5. Gut microbiota from BWBDS-treated mice independently alleviates SILI*

We next investigated the crosstalk between the intestinal flora and the anti-sepsis effects by performing an FMT experiment (Supporting Information Fig. S12B). We found higher survival rates in BWBDS feces recipients than CLP feces recipients post CLP (Fig. 4A) and there was no difference in body weight before CLP (Supporting Information Fig. S8A). Moreover, BWBDS feces recipients exhibited a trend towards higher serum IL-10 levels but there were no different in IL-6, IL-1β, and TNF-α secretion (Fig. 4B). Further, there were obvious differences in hepatic pathology injury in FMT mice by histological analysis. CLP feces recipients had higher histological scores than BWBDS feces recipients after CLP (Fig. 4C and D). In addition, BWBDS feces treatment due to the diminished proliferation of F4/80+CD11b+CD45+ macrophages in the liver compared with those in CLP feces recipients at 24 h after CLP (Fig. 4E). We also found that ALT and AST levels in BWBDS feces recipients were markedly decreased compared with those in CLP feces recipients at 24 h after CLP (Fig. 4F and G). Moreover, BWBDS feces treatment significantly increased colonic mRNA levels of ZO-1 and occludin compared with CLP feces recipients (Fig. 4H and I). BWBDS feces treatment also markedly improved the kidney and lung pathology changes after subjecting to CLP (Supporting Information Fig. S8B and C). We further explored the intestinal flora between BWBDS feces recipients and CLP feces recipients at 24h after CLP. The beta diversity (abund\_jaccard) indicated that the composition of intestinal microbiota between BWBDS feces and CLP feces recipients were different (Fig. 4J). Interestingly, there were different in the phylum and genus diversity between these two group mice (Fig. 4K, Supporting Information Fig. S8D and E). We found the abundance of *Lactobacillus* was increased in BWBDS feces recipients than CLP feces recipients (Fig. 4K and L). These results revealed that gut microbiota from BWBDS-treated mice may independently alleviate SILI, which may be associated with the increment of *Lactobacillus* and the improvement of gut-barrier function.

*3.6. L. johnsonii treatment independently alleviates SILI*

At present, we found the abundance of *Lactobacillus* was increased in the BWBDS-treated mice and BWBDS feces-treated mice. We guessed that *Lactobacillus* may be an important regulatory factor of the host response to sepsis-induced liver injury. To verify our hypothesis, we detected the potent activity of *Lactobacillus* species in LPS-stimulated RAW264.7 cells. We found that *L. johnsonii* showed most potent activity for induction of IL-10 secretion but had no effect on the induction of IL-6, IL-1β, and TNF-α (Supporting Information Fig. S9). We next investigated whether oral administration of *L. johnsonii* could reverse SILI *in vivo*. *L. johnsonii* was pretreated to mice before CLP surgery (Supporting Information Fig. S12C). Oral treatment with *L. johnsonii* significantly prolonged the survival rate after CLP. Moreover, *L. johnsonii* treatment group exhibited higher survival rate compared with BWBDS treatment group (Fig. 5A). Additionally, the levels of IL-6, IL-1β, and TNF-α in the serum of *L. johnsonii* treatment group were markedly diminished and the levels of IL-10 were significantly upregulated compared with CLP control mice (Fig. 5B-E). Moreover, the IL-6, TNF-α, IL-1β, CCL2, CCL3, CCL7, CXCL1 and CXCL10 mRNA were also obviously decreased in liver after *L. johnsonii* treatment compared with CLP control group (Fig. 5F). Histological analysis showed that livers from *L. johnsonii* treatment group had fewer pathological changes than CLP control group (Fig. 5G and H). As expected, decreased proportions of F4/80+CD11b+CD45+ macrophages in the liver were found in the *L. johnsonii* treatment group (Fig. 5I). We also found that the levels of ALT and AST in *L. johnsonii* treatment group were markedly diminished compared with those in CLP control group (Fig. 5J and K). The *L. johnsonii* treatment also markedly increased colonic mRNA levels of ZO-1 and occludin (Fig. 5L and M). These results demonstrated that *L. johnsonii* may independently alleviate sepsis-induced liver damage. Likewise, these results indicated that the effects of *L. johnsonii* alleviated SILI was consistent with that of BWBDS. These results suggested that the effects of BWBDS alleviated SILI may be mediated by the increasement of *L. johnsonii.*

*3.7. The anti-SILI effect of L. johnsonii treatment is related to promoting the anti-inflammatory activation of macrophages*

To determine the effects of the *L. johnsonii* therapy on the macrophages, we also analysed the immunological changes in the PMs, spleen, and pPBMC. Flow cytometry results showed that CLP induced a remarkable addition in the proportion of macrophages (F4/80+CD11b+CD45+) in PMs, spleen and pPBMC, which was reversed by *L. johnsonii* treatment (Fig. 6A). Further, we found thatM1 macrophages (CD80+F4/80+CD11b+) decreased meanwhile M2 macrophages (CD206+F4/80+CD11b+) increased in *L. johnsonii* treatment group compared with CLP control group (Fig. 6B and C). The production of functional cytokines, TNF-α and iNOS among M1 macrophages also decreased (Fig. 6D and E). In addition, we also observed the upregulation of IL-10 among M2 macrophages after *L. johnsonii* treatment (Fig. 6F). These results indicated that *L. johnsonii* treatment may take effect by activating M2 macrophages and suppressing the function of M1 macrophages, which were consistent with those of BWBDS. These results suggested that the effects of BWBDS promoting anti-inflammation function of macrophages may be mediated by *L. johnsonii.*

*3.8. L. johnsonii improves sepsis-induced liver injury dependent on macrophage participation*

To verify whether the protective effect of *L. johnsonii* is related to macrophages, Clo-Lipo was administered intraperitoneally to mice one day before CLP to deplete macrophages (Supporting Information Fig. S12D). Intraperitoneal injection of Clo-Lipo reduced the proportion of peripheral and circulatory macrophages and there was no different when compared with Clo-Lipo plus *L. johnsonii* treatment group (Fig. 7A). In addition, we found Clo-Lipo plus *L. johnsonii* treatment also decreased IL-6, TNF-α, IL-1β, CCL2, CCL3, CCL7, CXCL1, CXCL10 mRNA in the liver when compared with PBS-Lipo control group (Fig. 7B). Further, Clo-Lipo plus *L. johnsonii* treatment group had minor livers damage than PBS-Lipo control group by histological analysis (Fig. 7C and D). Moreover, reduced proportions of F4/80+CD11b+CD45+ macrophages were found in the liver of Clo-Lipo treatment group and Clo-Lipo plus *L. johnsonii* treatment group (Fig. 7E). Clo-Lipo plus *L. johnsonii* treatment *also* decreased the ALT and AST levels in the serum (Fig. 7F and G) and increased colonic ZO-1 and occludin mRNA compared with the PBS-Lipo control mice but showed no statistical difference when compared with the Clo-Lipo treated mice (Fig. 7H and I). The Clo-Lipo plus *L. johnsonii* treatment also reduced the protein level of IL-10 in the serum (Supporting Information Fig. S10A). Kidneys, lungs, and ileums pathological changes were improved in Clo-Lipo plus *L. johnsonii* treatment group (Supporting Information Fig. S10B-E). However, there were no significant differences in blood and PLF bacterial count between Clo-Lipo treatment group and PBS-Lipo control group (supporting information Fig.S10F and G). Overall, our data indicated that the macrophage may be one of key cells that mediate the protective effect of *L. johnsonii* on SILI.

*3.9. L. johnsonii regulates IL-10+M2 macrophages to alleviate SILI*

Since M2-like macrophages are resistant to necroinflammation triggered by necroptotic hepatocytes. Nevertheless, this hepatoprotection can be abolished by the application of an IL-10 antibody[30](#_ENREF_30). To investigate whether the liver protective effect of *L. johnsonii* is mediated by M2 macrophages, we used IL-10 antibody blockers to further investigate. Anti-IL-10R mAb was used to blocking IL-10 binding *in vivo* (Supporting Information Fig. S12E). As shown in (Fig. 8A), *L. johnsonii* treatment significantly increased IL-10+ M2 macrophages proportion in the PMs, spleen and pPBMC. We observed that *L. johnsonii* treatment also significantly increased colonic IL-10 mRNA and serum protein levels of IL-10 while in anti-IL-10R mAb mice were drastically reduced (Fig. 8B and C). *L. johnsonii* treatment significantly decreased the mRNA levels of IL-6, TNF-α, IL-1β, CCL2, CCL3, CCL7, CXCL1, CXCL10 in PLF compared with CLP control mice (Supporting Information Fig. S11A), while in *L. johnsonii* plus anti-IL-10R mAb treatment group had no obvious changes compared with CLP control group (Fig. 8D and Supporting Information Fig. S11B). Moreover, decreased proportion of F4/80+CD11b+CD45+ macrophages in the liver were not found in *L. johnsonii* plus anti-IL-10R mAbtreatment group compared with CLP control group (Fig. 8E). *L. johnsonii* treatment significantly reduced livers and ileums damage and histological scores, serum ALT and AST levels, and enhanced colonic ZO-1 and occludin mRNA compared with the CLP control group. However, *L. johnsonii* plus anti-IL-10R mAb treatment had no significant changes compared with CLP control group (Fig. 8F-K and Supporting Information Fig. S11C and D). These results indicated thatthe anti-sepsis effects of *L. johnsonii* may be, at least in part, dependent on IL-10+ M2 macrophages promotion.

*3.10. HI-L. johnsonii treatment alleviates SILI*

The cell surface component like lipoteichoic acid of *Lactobacillus* are important for strengthening the gut barrier and promoting IL-10 production in macrophages [31](#_ENREF_31),[32](#_ENREF_32). To further elucidate the treatment mechanism of *L. johnsonii,* we compared the treatment effect of live and death *L. johnsonii* on sepsis (Supporting Information Fig. S12F). Our results showed that oral treatment with HI*-L. johnsonii* significantly prolonged the survival rate after CLP. Moreover, HI-*L. johnsonii* mice seemed to have a higher survival rate than compared with live *L. johnsonii* (L-*L. johnsonii*) (Fig. 9A). Additionally, the levels of IL-6, IL-1β, and TNF-α in the serum of HI-*L. johnsonii treatment* group were markedly diminished and the levels of IL-10 was significantly upregulated compared with CLP control group (Fig. 9B-E). Reduced levels of ALT and AST were found in the serum of HI-*L. johnsonii* treatment group (Fig. 9F and G). Further, theHI-*L. johnsonii* treatment increased colonic mRNA levels of ZO-1 and occludin (Fig. 9H and I). Decreased proportion of total macrophages and increased IL-10+ M2 macrophages were found in the liver of HI-*L. johnsonii* treatment group and L-*L. johnsonii* treatment group (Fig. 9J and K). These results indicated that death bacteria also exerted beneficial effects on SILI, suggesting that cell membrane component of *L. johnsonii* provided beneficial effect. The cell membrane component of *L. johnsonii* maydirectly affect macrophages anti-inflammation function.

1. **Discussion**

Liver dysfunction and acute liver injury in patients with severe sepsis suggest a poor prognosis for clinical patients. Antibiotics is promising for sepsis treatment. However, the side-effects need to be improved. The main components of BWBDS such as amygdalin, cryptochlorogenic acid, nobiletin have been reported to have anti-inflammatory potential in inhibiting IL-1β, IL-6, TNF-α and relate to signal pathways like PI3K-Akt, MAPK[33-38](#_ENREF_33). Recent studies showed that administration of *ginseng* have been shown to increase the abundance of *Lactobacillus*[*39*](#_ENREF_39)*,*[*40*](#_ENREF_40)*.* These probioticswere reported to play an ameliorating role in sepsis, radioprotective, and intestinal ischemia/reperfusion (I/R) disease[41-43](#_ENREF_41). Due to difficult of fecal collection in mice after CLP surgery, we used preoperative fecal samples for FMT. However, FMT showed that the level of *L. murinus* rather than *L. johnsonii* was significantly increased in preoperative feces of BWBDS mice compared with the CLP mice. This was consistent with the report (https://www.shorelinebiome.com). In the present study, we first observed that the effect of the BWBDS alleviating SILI was attributable to the enhanced M2 macrophages and the downregulation of M1 macrophages by altering the gut microbiome.

Previous studies showed that different groups got different microbes associated with the effect on SILI. Whether it is related to *L. johnsonii* differences remains to be seen. Recently studies revealed that *L. johnsonii* exerts beneficial effects on disease-associated inflammatory response. Chen et. al. demonstrated that *L. johnsonii* reduces the levels of IL-6, TNF-α, and IL-1β to alleviate *Salmonella Typhimurium* (*S. Typhimurium*)-induced tight junction injury[44](#_ENREF_44). In addition, *L. johnsonii* have a protective effect on *Campylobacter jejuni* (*C. jejuni*)*-*induced colonic apoptosis[45](#_ENREF_45). Moreover, a study reported that *L. johnsonii* reduces alcohol-induced liver injury *in vitro* and *in vivo*[46](#_ENREF_46). Here, we first examined the influence of the gut microbiota *L. johnsonii* on the effect of SILI. These results revealed that enrichment of *L. johnsonii* in BWBDS treatment group may improve the SILI. *L. johnsonii* is a specie of commensal healthy bacteria[47](#_ENREF_47).

Microbial cell membrane intermediates the communication between microbiota and immune cells. *L. johnsonii* may be biologically active after heat treatment, although knowledge of the molecular mechanisms is lacking. Joo et. al.observed that macrophages treated with LPS in the presence of HI-*L. johnsonii* significantly decrease the productions of IL-6, IL-1β, and TNF-α compared with LPS alone[48](#_ENREF_48). Additionally, another report showed that heat killed *Lactobacillus paracacei* KW3110 (KW3110) induces dectin‐2 gene expression in macrophages and activates macrophages to produce IL‐10, which may be related to the characteristic carbohydrate chain of KW3110[49](#_ENREF_49). Oral administration of HI-*L. johnsonii* significantly inhibits the production of IL-1β and TNF-α, and increases the production of IL-10 and CD206 in TNBS-induced colitis mice by suppressing LPS-induced NF-κB activation[50](#_ENREF_50). In our study, we found HI-*L. johnsonii* treatment also significantly decreased the proportions of total macrophages and increased the proportions IL-10+ M2 macrophages in the liver. However, relatively little research has focused on *L. johnsonii* cell membrane. We reason the cell surface architecture of *L. johnsonii* may mediate TLR2-dependent ERK activation to alleviate SILI and regulate macrophages response[51](#_ENREF_51),[52](#_ENREF_52). Further study will address whether the specific structure of *L. johnsonii* cell membrane is potentially therapeutically useful for sepsis and whether the immune response is associated with this effect.

Macrophages are potential therapeutic target for inflammatory diseases and cancer[53](#_ENREF_53). The alterations from M1 to M2 phenotype may alleviate sepsis damage[54](#_ENREF_54). A previous study revealed that ginsenoside Rg3 could decrease the proportion of macrophages and turn M1 to M2 phenotype in LPS-induced mice[55](#_ENREF_55). Zhu et. al*. also* observed that baicalin ameliorates experimental inflammatory bowel disease through polarization of macrophages to an M2 phenotype[56](#_ENREF_56). However, whether BWBDS exhibits a similar effect is unknown. Our study has filled this gap by showing BWBDS can concurrently turn M1 to M2 phenotype and play anti-inflammatory roles on SILI. These results are consistent with the reduced SILI and increased survival time. Collectively, BWBDS may improve the SILI via promoting the anti-inflammation function of macrophages.

Additionally, previous study reported that oral administration of *L. johnsonii* significantly inhibited the production of total macrophages and accelerated polarization of M1 to M2 macrophages in mice with colitis[50](#_ENREF_50). However, whether *L. johnsonii* exhibits a similar effect on SILI is unknown. Here, we first examined the function of macrophage in the *L. johnsonii* treatment mice with SILI. Our results were consistent with previous studies, suggesting that the BWBDS promoting the anti-inflammation function of macrophages at list in parts, was mediated by *L. johnsonii.* Nevertheless, whether the protection effects of *L. johnsonii* dependent on macrophages in sepsis are not fully clarified. It was reported that Clo-Lipo could eliminate macrophages and had a specific protective effect on the sepsis induced injury in mice[57](#_ENREF_57). In our study, decreased ALT and AST levels were observed in Clo-Lipo treatment group and Clo-Lipo plus *L. johnsonii* treatment group. Collectively, *L. johnsonii* may improve the SILI via macrophage participation.

Limet. al.demonstrated that *L. johnsonii* treatment accelerates polarization of M1 to M2 macrophages and increases IL-10 secretion in mice with TNBS-induced colitis[58](#_ENREF_58). According to a report, M2-like macrophages can ameliorate necrotic hepatocyte-induced necroinflammation. However, this hepatoprotective effect was abolished with the use of IL-10 antibodies. Thus, M2-like macrophages suppress necroinflammation through IL-10[30](#_ENREF_30). Based on these studies, we further investigated whether *L. johnsonii* could reverse the SILI via M2 macrophage. We found compared with anti-IL-10R mAb treatment alone and CLP control group, *L. johnsonii* plus anti-IL-10R mAb treatment showed no different on SILI in mice. These findings suggested that *L. johnsonii* may improve the SILI by promoting IL-10+ M2 macrophages activation.

Although our study was not performed in germ-free mice, limiting the role of the microbiome in the phenotypic causality observed here. In addition, sepsis is a short period challenge, the microbial status just before CLP and after CLP are both important and the microbial composition before CLP but after BWBDS treatment should be further detected. So far, the treatment of sepsis and septic shock has been largely limited to supportive strategies other than the use of antibiotics[59](#_ENREF_59). Since all clinical trials using a single inflammatory cytokine have failed, some researchers have proposed that treatments that create a physical barrier between the gut and liver may offer more effective preventive and therapeutic strategies[5](#_ENREF_5). To make this purpose, we believed that our data indicated that herbal formula BWBDS alleviated SILI by increasing the gut microbiota *L. johnsonii* and regulating macrophage anti-inflammatory activity may be a new strategy for treating sepsis.

1. **Conclusions**

Our study provides novel insight into the “gut-liver-immune axis” in sepsis. Our data showed that BWBDS alleviated SILI by increasing the gut microbiota *L. johnsonii,* helping maintain intestinal integrity and promoting macrophage anti-inflammatory activity in mice. Furthermore, *L. johnsonii* reversed SILI by enhancing intestinal integrity and promoting the IL-10+ M2 macrophage anti-inflammation activity. Since there is limited therapy that can reduce SILI, modulation of the macrophage via administration with prebiotics, target bacteria, may provide new treatment strategies to sepsis. Our findings revealed that BWBDS may be used as a prebiotic while the gut microbiota *L. johnsonii* may be used as a probiotic for sepsis patients to improve SILI.

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**Authors’ contributions**

EL-HL, XQF and JMH designed the study. XQF, JMH and CTM performed the experiments. EL-HL, XY, JXC, XJY, XXF, QBW, PYY, RZL, JMH and XQF analyzed the data. XQF and JMH carried out the 16S rRNA analysis. XQF, JMH and ZBJ carried out the FACS analysis. JXC and LZ carried out the network pharmacology analysis. EL-HL, XQF, JMH wrote the manuscript. All authors reviewed and approved the manuscript.

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**Ethics approval**

Ethics approval of animal studies were provided by Macau University of Science and Technology. All experiments were performed in compliance with institutional animal care guidelines and protocols approved by the committee.

**Conflict of interest**

The authors declare that they have no competing interests.

**Availability of data and materials**

The data presented in this study are available in GitHub: https://github.com/fxqsyf/Herbal-formula-BWBDS-alleviates-polymicrobial-sepsis-induced-liver-injury-via-increasing-the-gut-mic.git.

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**Figure legends**

**Graphical abstract**: BWBDS improves the polymicrobial sepsis-induced liver damage by reinstating the gut microbiota. BWBDS potentiated the antisepsis effect and alleviated sepsis-induced liver injury via regulating macrophage anti-inflammatory activity, which enhancing M2 (alternatively activated) macrophages function, increasing the production of IL-10 and reducing the effect of M1 (classically activated) macrophages, as well as decreasing the production of TNF-α, IL-1β and IL-6, which might be addressed by reshaping gut microbiota. BWBDS treatment enriched the abundance of *Lactobacillus* when administrated into CLP mice. Notably, *L. johnsonii* treatment upregulated the expression of epithelium protecting tight junction protein, like ZO-1 and occludin regulating macrophage anti-inflammatory activity. CLP, cecal ligation and puncture; TNF-α, tumor necrosis factor-α; IL-6, Interleukin-6; IL-1β, Interleukin-1beta; IL-10, Interleukin-10; ZO-1, zonula occluden-1; *L. johnsonii*, *Lactobacillus johnsonii*; TJ, tight junction; CCL2, C-C motif chemokine ligand 2; CCL3, C-C motif chemokine ligand 3; CCL7, C-C motif chemokine ligand 7; CXCL1, C-X-C motif chemokine ligand 1; CXCL10, C-X-C motif chemokine ligand 10.

**Figure 1** BWBDS protects mice against sepsis-induced liver injury. (A) Survival rate. (B) Serum IL-6, (C) Serum IL-1β, (D) Serum TNF-α and (E) Serum IL-10 level. (F) IL-6, TNF-α, IL-1β, CCL2, CCL3, CCL7, CXCL1, CXCL10 mRNA levels of liver. (G) H&E staining (400x) and (H) histological score of livers to visualize the histomorphological features and quantitation analysis. (I) The proportion of F4/80+CD11b+CD45+ macrophage in liver. (J, K) Serum transaminase levels ALT and AST. (L, M) The relative mRNA levels of ZO-1 and occludin in the colon. n=9-11. Scale bar=100 μm. Error bars represent the mean ± SEM. Body weight curves were assessed by two-way ANOVA. Log-rank (Mantel-Cox) tests were performed for survival data. Other data were assessed by one-way ANOVA. (\*P<0.05, \*\*p<0.01, \*\*\*p<0.001; ns, nonsignificant).

**Figure 2** BWBDS decreases the M1 macrophages population and increases the M2 macrophages population after CLP. (A) PMs, spleen and pPBMC were analyzed by flow cytometry for (F4/80+CD11b+CD45+) macrophages cells in mice in the sham group, CLP vehicle group and BWBDS + CLP group. (B-F) M1 macrophages, M2 macrophages, expression of TNF-α, iNOS among M1 macrophages and IL-10 among M2 macrophages in the PMs, spleen and pPBMC. Each symbol represents an individual animal. n=6. Data represent the mean ± SEM and were analyzed by one-way ANOVA. (\*P<0.05, \*\*p<0.01, \*\*\*p<0.001).

**Figure 3** BWBDS-treated mice have unique intestinal microbiological composition. (A) Observed features in different groups. (B) Shannon index in different groups. (C) The level of *Firmicutes* in the feces. (D) The level of *Bacteroidetes* in the feces. (E) The *Firmicutes/Bacteroidetes* ratio in the feces. (F) The level of *Proteobacteria* in the feces. (G) Beta diversity analysis in different groups mice. (H) Relative abundance of top 15 genera in different treatment groups. (I) LEfSe analysis for differential abundant taxa detected between BWBDS + CLP group and CLP control group. Threshold parameters were set as p=0.05 for the Mann-Whitney U test and multiclass analysis=all against all. LDA score >2.0. n=8. The results are expressed as the mean ± SEM and were determined using two-tailed Student’s t test or one way ANOVA. (\*P<0.05, \*\*p<0.01, \*\*\*p<0.001; ns, nonsignificant).

**Figure 4** Gut microbiota from BWBDS-treated mice can independently alleviate sepsis-induced liver damage. (A) Survival rate. (B) Serum IL-6, IL-1β, TNF-α and IL-10 level of the feces recipient mice. (C, D) H&E staining (400x) and histological score of livers in different feces recipient mice. (E) The proportion of F4/80+CD11b+CD45+ macrophage in liver. (F, G) Serum transaminase levels ALT and AST. (H, I) The relative mRNA levels of ZO-1 and occludin in the colon. (J) Beta diversity analysis (abund\_jaccard). (K) Relative abundance of top 15 genera in different feces recipient mice. (L) LEfSe analysis for differential abundant taxa detected between BWBDS and CLP feces recipient mice. n=5. Scale bar=100 μm. The results are expressed as the mean ± SEM. Log-rank (Mantel-Cox) tests were performed for survival data. Other data were assessed by one-way ANOVA. (\*P<0.05, \*\*p<0.01, \*\*\*p<0.00; ns, nonsignificant).

**Figure 5** *L. johnsonii* improves sepsis-induced liver injury. (A) The survival rates. (B-E) Serum IL-6, IL-1β, TNF-α, and IL-10 level. (F) IL-6, TNF-α, IL-1β, CCL2, CCL3, CCL7, CXCL1, CXCL10 mRNA levels of liver. (G, H) H&E staining (400x) and histological score of livers. (I) The proportion of F4/80+CD11b+CD45+ macrophage in liver. (J, K) Serum transaminase levels ALT and AST. (L, M) The relative mRNA levels of ZO-1 and occludin in the colon was measured. n=8. Scale bar=100 μm. The results are expressed as the mean ± SEM. Log-rank (Mantel-Cox) tests were performed for survival data. Other data were assessed by one-way ANOVA. (\*P<0.05, \*\*p<0.01, \*\*\*p<0.001; ns, nonsignificant).

**Figure 6** *L. johnsonii* decreases the M1 macrophages population and increases the M2 macrophages population. (A) PMs, spleen and pPBMC macrophages were analyzed by flow cytometry for (F4/80+CD11b+CD45+)macrophages cells in different group mice. (B-F) M1 macrophages, M2 macrophages, and expression of TNF-α, iNOS among M1 macrophages and IL-10 among M2 macrophages in the PMs, spleen and pPBMC. Each symbol represents an individual animal. n=5. Data represent the mean ± SEM and were analyzed by one-way ANOVA or two-way ANOVA. (\*P<0.05, \*\*p<0.01, \*\*\*p<0.001).

**Figure 7** *L. johnsonii* improves sepsis-induced liver injury dependent on the participation of macrophages. (A) PMs, spleen and pPBMC were analyzed by flow cytometry for (F4/80+CD11b+CD45+) cells in mice. (B) IL-6, TNF-α, IL-1β, CCL2, CCL3, CCL7, CXCL1, CXCL10 mRNA levels of liver. (C, D) H&E staining (400x) and histological score of livers. (E) The proportion of F4/80+CD11b+CD45+ macrophage in liver. (F, G) Serum transaminase levels ALT and AST. (H, I) The relative mRNA levels of ZO-1 and occludin in the colon. n=5. Scale bar=100 μm. The results are expressed as the mean ± SEM and were determined by one-way ANOVA and Log-Rank test (B). (\*P<0.05, \*\*p<0.01, \*\*\*p<0.001; ns, nonsignificant).

**Figure 8**  *L. johnsonii* regulates M2 macrophages to alleviate sepsis-induced liver injury. (A) The proportion of IL-10+M2 macrophages were analyzed by flow cytometry in PMs, spleen and pPBMC in group mice. (B) The relative serum protein levels of IL-10. (C) Colonic mRNA levels of IL-10. (D) IL-6, TNF-α, IL-1β, CCL2, CCL3, CCL7, CXCL1, CXCL10 mRNA levels of liver. (E) The proportion of F4/80+CD11b+CD45+ macrophage in liver. (F, G) HE staining (400x) and the quantitation analysis of livers. (H, I) Serum transaminase levels ALT and AST. (J, K) The relative mRNA levels of ZO-1 and occludin in the colon. n=5. Scale bar=100 μm. The results are expressed as the mean ± SEM and were determined by one-way ANOVA or two-way ANOVA. (\*P<0.05, \*\*p<0.01, \*\*\*p<0.001; ns, nonsignificant).

**Figure 9** HL-*L. johnsonii can* improve sepsis-induced liver injury. (A) The survival rates. (B-E) Serum IL-6, IL-1β, TNF-α, and IL-10 level. (F, G) Serum transaminase levels ALT and AST. (H, I) The relative mRNA levels of ZO-1 and occludin in the colon was measured. (J, K) The proportion of total macrophage and IL-10+M2 macrophages in liver were analyzed by flow cytometry in liver. n=8. The results are expressed as the mean ± SEM. Log-rank (Mantel-Cox) tests were performed for survival data. Other data were assessed by one-way ANOVA. (\*P<0.05, \*\*p<0.01, \*\*\*p<0.001; ns, nonsignificant).

1. *Abbreviations***:** SILI, Sepsis-induced liver injury; ICUs, Intensive care units; CLP, Cecal ligation and puncture; CS, Cefoxitin sodium; FMT, Fecal microbiota transplantation; SMRT, Single-molecule real-time; Anti-IL-10R mAb, Anti-interleukin-10 receptor mouse antibody; *L. johnsonii, Lactobacillus johnsonii;* PCR, Polymerase chain reaction; PMs, Peritoneal macrophages; pPBMC, Polarized peripheral blood mononuclear cells; INOS, Inducible nitric oxide synthase; IL-6, interleukin-6; IL-1β, Interleukin-1β; TNF-α, Tumor necrosis factor; PIK3CA, Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha; AKT1, AKT serine/threonine kinase 1; PIK3R1, Phosphoinositide-3-kinase regulatory subunit 1; APP, Amyloid precursor protein; MAPK1, Mitogen-activated protein kinase 1; EP300, E1A binding protein P300; MAPK3, Mitogen-activated protein kinase 3; STAT3, Signal transducer and activator of transcription 3; UBC, Ubiquitin C; SRC, sarcoma; KEGG, Kyoto Encyclopedia of Genes and Genomes; GO, Gene ontology; LEfSe, Linear discriminant analysis effect size; LPS, Lipopolysaccharide; OUT, Operational taxonomic unit; PCoA, Principal coordinate analysis; PCA, Principal component analysis; PICRUSt, Phylogenetic Investigation of Communities by Reconstruction of Unobserved States; IL-17, Interleukin-17; PI3K-Akt, Phosphatidyl-inositol 3-kinase/serine-threonine kinase; MAPK, Mitogen-Activated Protein Kinase 3; CCL, C-C motif chemokine ligand; CXCL, C-X-C motif chemokine ligand; ERIC, Enterobacterial repetitive intergenic consensus; ZO-1, Zonula occludens-1; Clo-Lipo, Clodronate liposomal; HI-*L. johnsonii*, Heat inactivation *L. johnsonii;* L-*L. johnsonii*, Live *L. johnsonii.* [↑](#footnote-ref-1)